CHANGES IN TRYPSIN AND CHYMOTRYPSIN OCCURING DURING STORAGE OF SHEEP PANCREATA

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ABSTRACT

The possibility that endogenous sheep insulin may be degraded by the proteolytic enzymes of pancreata during storage and processing has been investigated. Chilled pancreata contained little proteolytic activity after 3 days (trypsin : 42 u. kg^{-1}). The release of proteases from thawed tissue was also slow (trypsin 1,300 u. kg^{-1}). ^{125}I-insulin was degraded by only 14% after 72 hours exposure to pancreas homogenate. These observations suggest that sheep insulin is not destroyed by proteases if the pracreata are stored under the usual low temperature conditions.

INTRODUCTION

As the medical sophistication of both the developed and developing countries increases, the known incidence of diabetes is increasing (Eli Lilly & Co. 1973). This, in turn, is causing an increase in demand for insulin. Mammalian insulins differ in composition from human insulin in only a few non-significant amino acids (Smith, 1966) and can be used for the treatment of diabetes as a replacement for the missing human insulin. The usual source of insulin for the treatment of this disease is beef or pig pancreas.

Every year in New Zealand a large number of sheep and lambs (35 - 40,000) are slaughtered and the pancreata from these animals are used, if at all, only for low value products such as pet food. Very few are used for the production of insulin.

It has been reported that the recovery of insulin from sheep pancreata is very low (300- 1,000 i.u. kg^{-1} per gland*) (Swan, 1971). Available data on the recovery of sheep insulin do not allow a definite conclusion to be drawn. Possibly
1) a low content of insulin in the sheep pancreas
2) the use of poor extraction techniques

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* Pure insulin is measured in internation units (i.u.) with 24 i.u. g^{-1}.

3) the rapid degradation of the insulin during the processing, may be the cause of the low insulin recovery.

Degradation of insulin (3) could be brought about by the action of proteolytic enzymes such as trypsin and chymotrypsin. These proteases, found in the prancreas as inactive trypsinogen and chymotrypsinogen, may be released from zymogens during the handling process or storage. We have investigated this by measuring the activity of trypsin and chymotrypsin extracted from pancreata subjected to a variety of storage treatments. From these experiments we have been able to recommend optimum storage conditions to reduce proteolysis and thereby retain the maximum recoverable insulin.

MATERIALS AND METHODS

Pancreata were collected at the freezing works (Waitaki-New Zealand Refrigeration Co., Islington) from freshly killed sheep or lambs and either stored on ice or frozen immediately.

For enzyme extraction the pancreata were trimmed of excess fat where possible and homogenised in 0.5 M-NaCl.0.02 M-$NaHCO_3$ pH 8.0 (100 ml/100g). The homogenate was centrifuged at 4,000 X gravity at 3°C and the supernatant was used as the source of enzymes.

AFFINITY CHROMATOGRAPHY

Soy bean anti-trypsin (100mg) was coupled to 15 g of CNBr-Sepharose (Pharmacia) at pH 8.5 following the general method of Cuatrecasas (1970).

A column of the anti-trypsin-Sepharose (STI-Sepharose) 8 X 100 mm was equilibrated with 0.5 M-NaCl, 0.02 M-$NaHCO_3$, pH 8.0 buffer and charged with 18 - 20 ml of the pancreas enzyme supernatant. The column was washed with a suitable volume of pH 8.0 buffer until protein elution ceased (approx. 50 ml). The proteolytic enzymes were then eluted with 0.2 M-KCl, 0.02 M-HCL pH2.02. The active fractions were pooled, dialysed against distilled water and lyophilised for storage.

ION-EXCHANGE CHROMATOGRAPHY (Feinstein *et al.* 1974)

The active enzyme fraction from the affinity column was dissolved in 0.02 M-Na acetate (pH 4.0) containing 0.02 M-$CaCl_2$ and poured onto a column of SP-Sephadex C-25 (9 X 150mm). The column was eluted with 30ml of pH 4.0 buffer, followed by 90 ml of 0.02 M-NaOAc, plus 0.02 M-$CaCl_2$ (pH 5.0) and 100 ml of pH 5.0 buffer containing 0.2 M-NaCl.

ENZYME ASSAYS

Trypsin was assayed spectrophotometrically using Tosyl-arginine methyl ester (TAME) (Walsh 1970) and chymotrypsin with Benzyl-Tyrosine ethyl ester (BTEE) (Walsh and Wilcox 1970).

GEL FILTRATION OF INSULIN (Epstein and Anfinsen 1963)

^{125}I-insulin (The Radiochemical Centre, Amersham, England) was purified by gel-filtration on Sephadex G.50 equilibrated with 0.2 M-NH_4HCO_3 pH 7.8 and eluted with the same buffer. The insulin fractions were pooled, dialysed against distilled water and redissolved in 0.02 M-$NaHCO_3$ pH 8.0.

The purified ^{125}I-insulin (25,000 dpm) was incubated with prancreas homogenate and re-isolated from this by gel filtration (Fig. 1). The radioactive insulin fractions were pooled and assayed for insulin using the insulin antibody technique of Midgeley, Rebar and Niswender (1969).

^{125}I was counted in a Packard Model 3001 Tri-Carb scintillation spectrophotometer.

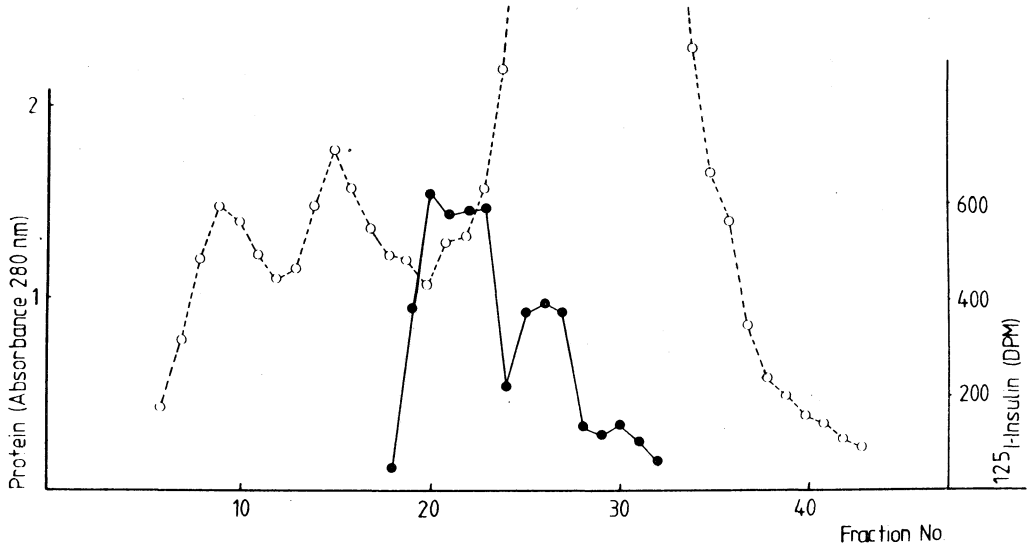

Fig. 1. Gel filtration on Sephadex G-50 of ^{125}I-insulin after incubation with homogenised pancreas. Fraction size 5 ml. Absorbance at 280 nm (0-0). Radioactivity (dpm) (0-0).

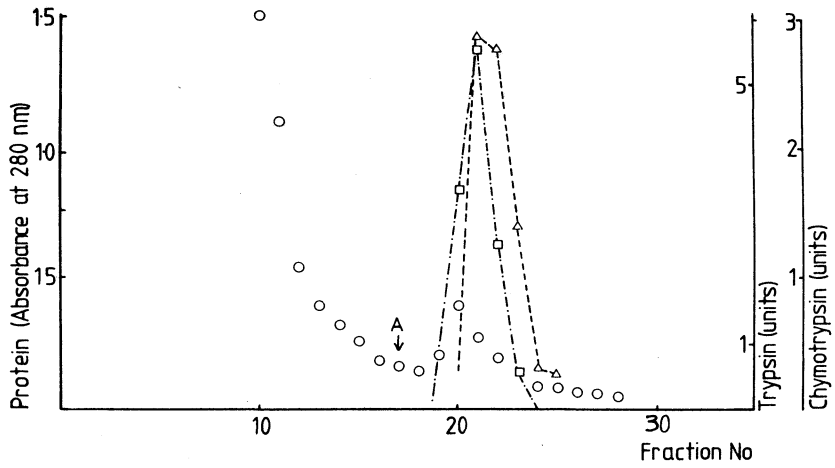

Fig. 2. Isolation of proteolytic enzymes from freshly frozen prancreata by affinity chromatography. Fraction size 3 ml. Absorbance at 280 nm (000); Enzyme activity against TAME (trypsin) (Δ Δ); Enzyme activity against BTEE (chymotrypsin) (□ □).

RESULTS AND DISCUSSION

PROTEOLYTIC ACTIVITY

The trypsin and chymotrypsin of sheep pancreas homogenates can be separated from the bulk of the protein by affinity chromatography (Fig. 2). Further chromatography of the active material on SP-Sephadex C-25 shows that both enzymes are present in a number of iso-zymic forms (Fig. 3a). If the pancreas is allowed to autolyze for 24 h at 3°C (Fig. 3b), the total proteolytic activity increases as the zymogens are hydrolysed but there is little evidence of a change in the distribution of activity.

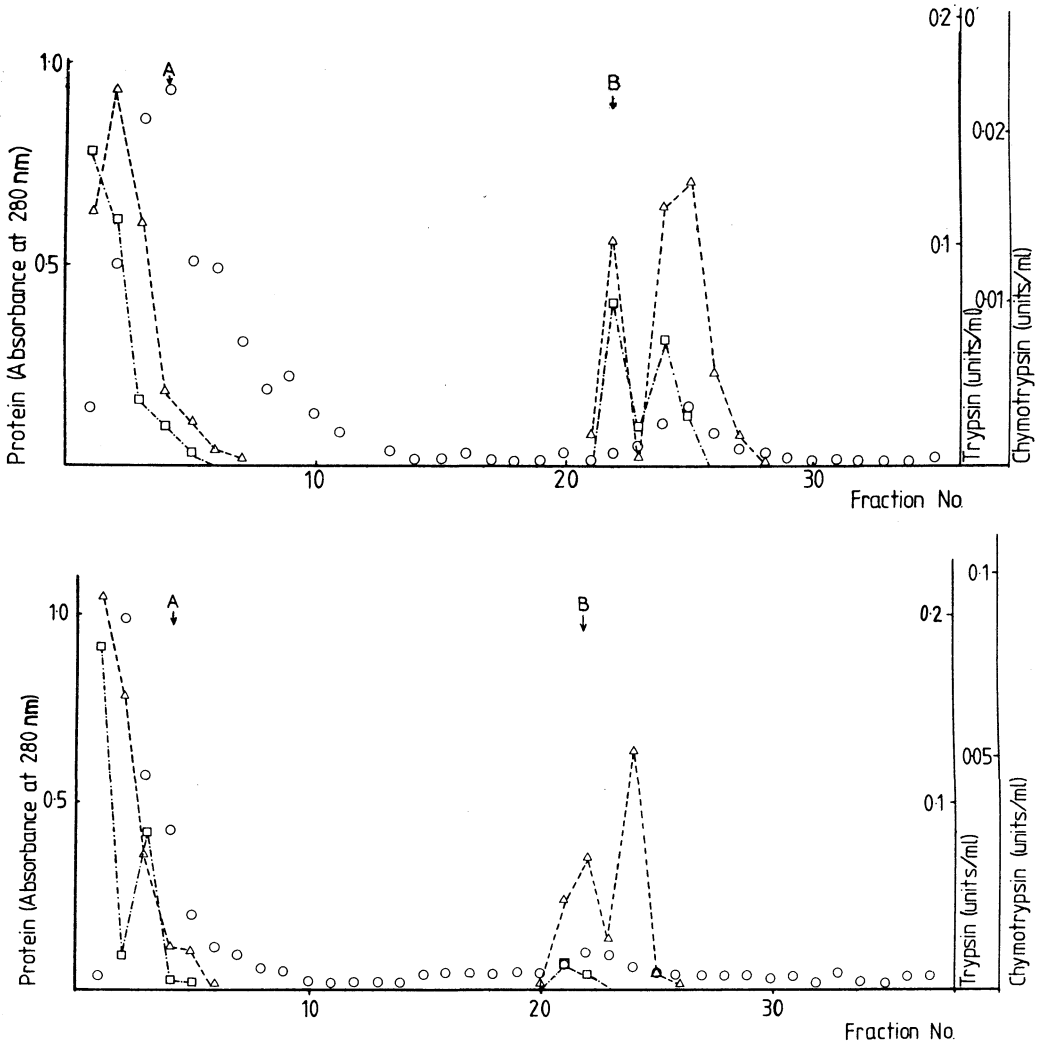

Fig. 3. Elution of partially purified hydrolytic enzymes from SP-Sephadex C-25.

a) enzyme preparation derived from frozen pancreas
b) enzyme preparation from pancreas autolysed for 24 hours.

Buffer changes at points A and B. Fraction size 5 ml. Symbols as in Fig. 2.

QUANTITIVE CHANGES

For the direct assay of trypsin and chymotrypsin activity in pancreata it was found to be necessary to separate the proteolytic enzymes by affinity chromatography. The activity of the enzymes from pancreata subjected to a variety of storage conditions is listed in Table 1, where it may be seen that proteolysis in

the pancreas is at a very low level if the gland is stored on ice or frozen. Significant proteolysis occurs if the chilled gland is stored for three days or more, or if the frozen pancreas is allowed to thaw. This latter is probably due to the breakdown of the tissue during the freezing and thawing.

TABLE 1. PROTEOLYTIC ACTIVITY RELEASED FROM SHEEP PANCREAS.

1 unit of trypsin is equivalent to the hydrolysis of 1 m TAME min. $^{-1}$.
1 unit of chymotrypsin is equivalent to the hydrolysis of 1 m BTEE min $^{-1}$.

Treatment	(u. kg^{-1} fresh weight) Trypsin	Chymotrypsin
1) Pancreas frozen immediately after killing		
Ewe		
a) Frozen pancreas	30-60	3-20
b) Thawed 1.5 h	68	-
c) Thawed 16h	1300	1310
Lamb		
d) Frozen pancreas	14	-
e) Thawed 3 h	55	-
2) Chilled pancreas (Ewe)		
a) Stored at 0° for 1 h	0.6	14
b) Stored 24 h	4	18
c) Stored 3 days	42	45
3) Homogenised pancreas (Ewe)		
a) Autolysed for 12 h	35	51
48 h	6260	1860

TRYPSIN INHIBITOR

Extraction and assay of the endogenous trypsin inhibitor by the technique of Laskowski (1955) shows the presence of inhibitor at a level of 5 - 10 g inhibitor per kg fresh weight of gland. This inhibits 50 - 300 units of trypsin and therefore is adequate to control porteolysis in chilled pancreata.

INSULIN HYDROLYSIS

To check the stability of insulin in the presence of low levels of proteolytic enzymes in pancreata, ^{125}I- labelled insulin was incubated with homogenised pancreas. The destruction of insulin was determined by isolating undegraded insulin by gel chromatography and subsequent assay by precipitation with insulin antibody. As shown in Table 2 the recovery of insulin was only slightly affected by exposure to the homogenate, which again

suggests that little *in vivo* degradation occurred.

TABLE 2. DEGRADATION OF ^{125}I-INSULIN BY PANCREAS HOMOGENATES

	^{125}I dpm	% recovery
a) ^{125}I-insulin isolated immediately after mixing	58,762	98.1
b) ^{125}I-insulin isolated after 72 h	50,923	85.7

Our results suggest that sheep insulin is stable in the pancreas and during extraction when the normal precautions of chilling or freezing the glands and performing the extraction at 3°C or less are followed.

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